Australian National Algae Culture Collection - Methods

EXHIBIT A

Algal growth phases including determination of the growth rate and population doubling time

There are 5 reasonably well defined phases of algal growth in batch cultures (Fogg and Thake, 1987)

1 lag; 2 exponential; 3 declining growth rate; 4 stationary; 5 death. Each of the phases is described below and in Fig 1 (goto fig). Alternatively Jump straight to growth rate equation

Lag phase

The condition of the innoculum has a strong bearing on the duration of the lag phase (Spencer, 1954). An innoculum taken from a healthy exponentially growing culture is unlikely to have any lag phase when transferred to fresh medium under similar growth conditions of light, temperature and salinity. In general the length of the lag phase will be proportional to the length of time the innoculum has been in phases 3-5. A lag phase may also occur if the innoculum is transferred from one set of growth conditions to another.

Exponential phase and calculating growth rates

The growth rate of a microalgal population is a measure of the increase in biomass over time and it is determined from the exponential phase. Growth rate is **one** important way of expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed upon it. The duration of exponential phase in cultures depends upon the size of the innoculum, the growth rate and the capacity of the medium and culturing conditions to support algal growth. Biomass estimates need to be plotted over time, and logistical constraints determine their frequency but once every one to two days is generally acceptable. Cell count and dry weight are common units of biomass determination. *In-vivo* fluorescence and turbidity can be used as surrogate measures which enable higher temporal resolution due to the logistical ease of measurement (correlations between fluorescence or turbidity and cell count can be established but they will become less accurate as experimental conditions are varied. For example cell fluorescence may vary with temperature so an experiment with several test temperatures may need correlations to be determined for each temperature. Correlations also become innacurate as cultures move into stationary phase so fluorescence can not be used as a substitute for cell counts where an estimate of final cell yield is needed). Once the growth phase has been plotted (time on x-axis and biomass on logarthmic y-axis) careful determination of the exponential (straightline) phase of growth is needed. Two points, N1 and N2, at the extremes of this linear phase (see fig below) are taken

and substituted into the equation

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Growth rate ; K' = Ln (N2 / N1) / (t2 - t1)

Where N1 and N2 = biomass at time1 (t1) and time2 (t2) respectively; Levasseur *et al* (1993).

Divisions per day and the generation or doubling time can also be calculated once the specific growth rate is known.

Divisions per day ; $Div.day^{-1} = K' / Ln2$

Generation time ; $Gen' t = 1 / Div.day^{-1}$

For healthy cells of a robust species, small innoculums equal to 0.5 % of the volume of the new culture will normally generate new healthy cultures. If the species is delicate or the culture less healthy then a larger innoculum of ~ 10% may be needed to support a new culture. (Many of the stock cultures in CMARC are transferred with a 0.5 to 1 mL innoculum into 40 mL fresh medium representing a 1.25 % to 2.5% innoculum).

Declining growth

Declining growth normally occurs in cultures when either a specific requirement for cell division is limiting or something else is inhibiting reproduction. In this phase of growth biomass is often very high and exhaustion of a nutrient salt, limiting carbon dioxide or light limitation become the primary causes of declining growth. When biomass is increasing exponentially a constant supply of air (or air plus CO_2) will only be in balance with growth at one point during exponential phase. At low cell densities too much CO_2 may lower the pH and depress growth. CO_2 limitation at high cell densities causes any further biomass increase to be linear rather than exponential (with respect to time) and proportional to the input of CO_2.

Light limitation at high biomass occurs when the cells absorb most of the incoming irradiation and individual cells shade each other (hence the often quoted term “self-shading”). Growth in most phytoplankton is saturated at relatively low irradiances of 50-200 µmol. photons m^{-2} s^{-1} (cf noontime irradiance at the water surface in the tropics of 2000 µmol. photons m^{-2} s^{-1}). Microalgae are therefore generally well adapted to surviving conditions of low incident light and may survive for extended periods under these conditions.

Stationary phase

Cultures enter stationary phase when net growth is zero, and within a matter of hours cells may undergo dramatic biochemical changes. The nature of the changes depends upon the growth limiting factor. EXHIBIT A Nitrogen limitation may result in the reduction in protein content and relative or absolute changes in lipid and carbohydrate content. Light limitation will result in increasing pigment content of most species and shifts in fatty acid composition. Light intensities that were adequate or optimal for growth in the first 3 phases can now become stressful and lead to a conditon known as photoinhibition. It is important that while the measured light intensity within the culture will decrease with increasing biomass if the incident illumination is maintained relatively high then a large proportion of cells may become stressed, photoinhibit and the culture can be pushed into the death phase. This is especially the case if the culture is also nutrient stressed. It is preferable for many species to halve or further reduce the incident light intensity when cultures enter stationary phase to avoid photoinhibition. Some green algae and cyanobacteria may survive in the vegetative state (ie not as cysts) for over 6 – 12 months under very low illumination. For many species lower temperature combined with lower irradiance can further reduce stress. Survival is inversely proportional to temperature but only in darkness. Some algal species may form long lived cysts or temporary resting cysts with greatly reduced metabolism under different conditions of stress. The shut down of many biochemical pathways as stationary phase proceeds means that the longer the cells are held in this condition the longer the lag phase will be when cells are returned to good growth conditions.

Death phase

When vegetative cell metabolism can no longer be maintained the death phase of a culture is generally very rapid, hence the term “culture crash” is often used. The steepness of the decline is often more marked than that represented in the accompanying growth figure. Cultures of some species will lose their pigmentation and appear washed out or cloudy, whereas cells of other species may lyse (no recognizable cells) but the culture colour will be maintained. The latter is an important consideration and one reason why colour should not be relied upon to guage culture health. Bacteria which may have been kept in check during exponential and early stationary phase may “explode” as cell membrane integrity become progressivley compromised or leaky and a rich carbon source for bacterial growth is released. Free pigment and bacterial growth are further reasons why measures of turbidity or fluorescence should not be used beyond early stationary phase as surrogate biomass indicators, or especially as indicators of culture health. Occassionally cell growth of some species can reoccur after a culture has apparently died. In this instance most vegetative cells will have died, and possibly most of the bacteria, releasing nutrients back into the media. Then either the very few remaining vegetative cells or more likely germination of cysts or temporary cysts will be able to fund this secondary growth.

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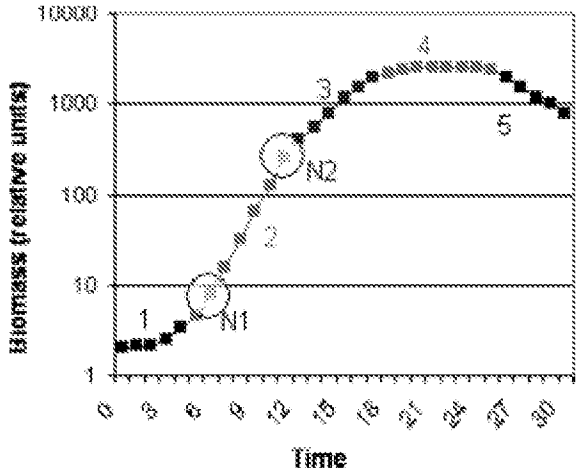

Fig 1 General pattern of microalgal growth in batch cultures

Specific Growth rate K'	Divisions per day Div.day^{-1}	Generation time = Doubling time days	hours	Specific Growth rate K'	Divisions per day Div.day^{-1}	Generation time = Doubling time days	hours
0.10	0.144	6.931	166.36	1.05	1.515	0.660	15.84
0.15	0.216	4.621	110.90	1.10	1.587	0.630	15.12
0.20	0.289	3.466	83.18	1.15	1.659	0.603	14.47
0.25	0.361	2.773	66.54	1.20	1.731	0.578	13.86
0.30	0.433	2.310	55.45	1.25	1.803	0.555	13.31
0.35	0.505	1.980	47.53	1.30	1.876	0.533	12.80
0.40	0.577	1.733	41.59	1.35	1.948	0.513	12.32
0.45	0.649	1.540	36.97	1.40	2.020	0.495	11.88
0.50	0.721	1.386	33.27	1.45	2.092	0.478	11.47
0.55	0.793	1.260	30.25	1.50	2.164	0.462	11.09
0.60	0.866	1.155	27.73	1.55	2.236	0.447	10.73
0.69	1.000	1.000	24.00	1.60	2.308	0.433	10.40
0.65	0.938	1.066	25.59	1.65	2.380	0.420	10.08
0.70	1.010	0.990	23.77	1.70	2.453	0.408	9.79
0.75	1.082	0.924	22.18	1.75	2.525	0.396	9.51

0.80	1.154	0.866	20.79	1.80	2.597	0.385	9.24
0.85	1.226	0.815	19.57	1.85	2.669	0.375	8.99
0.90	1.298	0.770	18.48	1.90	2.741	0.365	8.76
0.95	1.371	0.730	17.51	1.95	2.813	0.355	8.53
1.00	1.443	0.693	16.64	2.00	2.885	0.347	8.32

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